

**R E M A R K S**

The Office Action dated August 22, 2001 presents the examination of claims 6-9 and 12-13. Claims 6-8 are amended. No new matter is inserted into the application.

***Interviews***

Applicants' representative extends gratitude to the Examiner for the helpful interviews held on Wednesday, December 17, 2001, and Tuesday, January 22, 2002.

***Specification***

The Examiner objects to the specification because the abstract is not located on a separate sheet. In response to the Examiner's remarks, Applicants submit herewith an abstract on a separate sheet. Thus, the instant objection is overcome.

***Rejection under 35 U.S.C. § 102(b)***

The Examiner maintains the rejection of claims 6-9 and 12-13 under 35 U.S.C. § 102(b) for allegedly being anticipated by Nakao et al. (*Cancer Res.* 55:4248-4252). Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Nakao et al. discloses a peptide antigen expressed on SCC from KE-4 tumor cells. The Examiner asserts that the peptide antigens disclosed by Nakao et al. are the same peptide antigens of the present invention, absent evidence to the contrary. The Examiner also states that the declaration filed by Dr. Itoh is not persuasive. Specifically, the Examiner asserts,

"...the Declaration does not state that the protein of Nakao et al. in fraction number 23 is not that of amino acid sequence of SEQ ID NO:2 in the instant application." (page 3, lines 8-6 from the bottom), "...the response fails to show a clear distinction between the claimed protein and the protein of Nakao et al." (page 3, line 4-3 from the bottom), "the protein of Nakao et al. is isolated (HPLC fractionation) and the protein in fraction 23 would inherently contain the amino acid sequence of SEQ ID NO:2 and be encoded by SEQ ID NO:1: (page 4, line 4-6)."

Applicants respectfully disagree with the Examiner's assertions. First, the starting material of the HPLC fractionation is a mixture obtained by pre-fractionation and consisting of peptides of up to  $M_r$  3000 (about 30 amino acids). Specifically, Nakao et al. obtained the peptide fractions from peptides eluted from KE-4 cell using pH3.3 acid elution technique, comprising pretreating the extract with C18 Sep Pak, prefractionating with Centricon-3, and fractionating with reverse phase HPLC (see, pp.4248, right column, 5<sup>th</sup> full paragraph "Peptides of Tumor Cells", lines 1-7 of Nakao et al., *Cancer Research* 55, 4248-4252). The prefractionation could not yield a mixture of peptides having molecular weight of more than  $M_r$  3000 (more than about 30 amino acids), as can be

seen from the description "Cetricon-3" (Amicon. Beverly, MA; consisting of peptides  $\leq$  Mr 3000,  $\approx$  30 amino acids).

Thus, it is *prima facie* impossible that the protein of the present invention shown by SEQ ID NO:2 (which is composed of 800 amino acids) could be present in fraction 23 or any other fractionations disclosed by Nakao et al. As evidence thereof, Applicants submit a second declaration (attached hereto) by Dr. Itoh unequivocally stating that the protein in fraction 23 of Nakao et al. (or any other fraction) is not the instant polypeptide sequence.

During the interview held on December 17, 2001, the Examiner indicated that the statement that fraction 23 does not contain SEQ ID NO:2 was not sufficient for overcoming the prior art rejection. Specifically, the Examiner agreed that a protein comprising SEQ ID NO:2 or encoded by a DNA comprising SEQ ID NO:1 was not anticipated by Nakao et al. (i.e. sections (a) and (b) of claim 6). However, the Examiner insisted that a protein encoded by a DNA which hybridizes to the complement of SEQ ID NO:1 might contain 30 or less amino acids and as such could be present in fraction 23 of Nakao et al. For example, the Examiner stated that it is known in the art that the average number of amino acids that can bind to MHC class I antigen is 9 amino acids. Thus, the Examiner maintained that the protein of SEQ ID NO:2 could be

decomposed to a fragment that is small enough to be present in fraction 23 of SEQ ID NO:2.

In response to the Examiner's remarks, Applicants amend claim 6, section (c) to recite a protein of about 800 amino acids. Support for this amendment is found in the specification, particularly on page 33, lines 11-12, and in SEQ ID NO:2. The term "about" is used to clarify that the protein recited in (c) can consist of 800 amino acids, plus or minus 5% of the amino acids. During the interview held on January 22, 2002, the importance of the term "about" was stressed to the Examiner. The Examiner stated that since there was no direct support in the specification for the term, he was unsure whether it would be acceptable. However, Applicants strongly submit that a skilled artisan would clearly understand the meaning of "about." The skilled artisan would easily understand that the present invention is contemplated to include a protein defined by the amino acid sequence shown in SEQ ID NO:2 and also a variant thereof, in which one or more amino acid residues are substituted, deleted, or added, as long as the variant protein is capable of yielding, through its intracellular decomposition, peptide fragments which can bind to MHC class I antigen and which can be recognized by T cells in such binding state. See, for example, the paragraph bridging page 8 to page 9, and page 10, lines 11-24 of the specification.

The Examiner suggested during the interview that statements be made in the "Remarks" section of this Reply, specifying that one of skill in the art would understand that "about" is equivalent to 800 plus or minus 5% of amino acids. As Applicants have complied with the Examiner's suggestions, Applicants respectfully submit that the term "about" should not be considered new matter.

During the interview held on January 22, 2002, the Examiner tentatively agreed that the addition of the phrase "of about 800 amino acids which is" to claim 6 would overcome the anticipation rejection over Nakao et al., since Nakao et al. only discloses peptides of less than 30 amino acids. However, the Examiner also mentioned that he was unsure whether the starting material of Nakao et al. (i.e. the protein before fractionation) would consist of a peptide of about 800 amino acids. Even though Applicants believe that the burden is on the Examiner, rather than Applicants, to show or reason that an 800 amino acid peptide is inherent in the disclosure of Nakao et al., Applicants respectfully submit that there is no chance that the starting material of Nakao et al. contains the tumor antigen protein of the present invention as recited in the amended claim 6.

Specifically, the starting material of Nakao et al. is a bunch of peptides non-covalently bound to the cell surface of KE-4 cells (Applicants note that the KE-4 cell is not the starting material for

fractionation, but rather the peptides are the starting material). In contrast, the tumor antigen protein of the present invention as claimed in claim 6 is a protein that is never presented on the cell surface as it is. The tumor antigen protein of claim 6 yields a peptide fragment(s) to be presented on the cell surface only after it has undergone intracellular decomposition. It is well known in the art that a tumor antigen protein must be decomposed intracellularly to yield a peptide(s) that can be presented on the cell surface. The same is true for the tumor antigen protein recited in claim 6, sections (a), (b), and (c). This mechanism is described on page 2, lines 2-8 of the specification:

"Tumor antigen peptides are generated from tumor antigen proteins. Thus, the proteins are intracellularly synthesized and then degraded in cytoplasm into the peptides by proteasome. On the other hand, MHC class I antigens formed at endoplasmic reticulum bind to the above tumor antigen peptides, and are transported via cis Golgi to trans Golgi, i.e., the mature side, and expressed on the cell surface (Rinsho-Menneki, 27(9):1034-1042, 1995)."

This mechanism is also clearly shown in the literature, for example, in Boon et al., *Scientific American*, March 1993, pp. 39 (a copy of which is attached hereto as Exhibit 1). The Examiner's attention is drawn to the diagram on page 36, which shows how an antigenic protein is processed to the cell surface.

Accordingly, it is apparent that the starting material of Nakao et al. does not contain the tumor antigen protein of claim 6. Applicants

remind the Examiner that Nakao et al. obtained a peptide mixture comprising a peptide consisting of, at most, 30 amino acids (see "Peptides of Tumor Cells," page 4248, right column). As such, Nakao et al. fails to disclose or suggest the subject matter of claim 6.

The Examiner did not directly state that he would again reject claim 7 for being anticipated by Nakao et al. However, as above, the Examiner mentioned that he might issue an obviousness rejection, because although Nakao et al. does not directly disclose the claimed protein, said proteins allegedly may be inherently present in the fractionations or starting material disclosed by Nakao et al. Although an obviousness rejection has not been made, in the interest of compact prosecution Applicants respectfully respond. First, claim 7 is amended to recite to that tumor antigen protein is purified. Support for this amendment can be found throughout the specification, especially in the process for producing a peptide of the present invention as described on page 17, lines 12-24 (peptide synthesis), Example 4 on page 36, lines 11-22. Actually, the tumor antigen peptide fragment of the present invention was obtained by peptide synthesis and purification, as exemplified by the specification.

Second, Applicants respectfully submit that the fact that the peptide fragments recited in the amended claim 7 are purified clearly distinguishes claim 7 over Nakao et al. Specifically, Nakao et al. did

not isolate any of the peptides in the HPLC fractions. As such, the purification limitation of claim 7 excludes a fraction of peptides as is disclosed by Nakao et al.

Overall, Applicants submit that it is impossible for a skilled person to obtain the peptide fragment of claim 7 in accordance with the teaching of Nakao et al. as explained above and in Dr. Itoh's second Declaration, especially on pages 3 and 4. Further, KE-4 cell is a tumor cell established from an isolated cell originated from a particular tumor patient, and hence had not been publicly available before its deposition date (May 23, 1997). In addition, there are many (more than one thousand) peptides on a cell surface that can be presented to a sole HLA type. As evidence thereto, the Examiner's attention is drawn to Hunt et al. (*Science*, 225: 1261-1263, 1992), see Exhibit 2, pp. 1262, sp. as underlined. This means that for human beings having about six HLA types, there should be about 6000 different peptides presented on the cell surface, which in turn means that any respective fraction of Nakao's 50 fractions shown in Fig. 3 would theoretically contain at least 120 different peptides. Accordingly, the skilled artisan could not obtain the single peptide fragment as claimed in claim 7 each and every time in accordance with the teaching of Nakao et al.

For these reasons, Applicants respectfully submit that the present invention is neither anticipated by, nor obvious over, Nakao et al. Withdrawal of the instant rejection is requested.

***Rejection under 35 U.S.C. § 112, first paragraph***

The Examiner rejects claims 6-9 and 12-13 under 35 U.S.C. § 112, first paragraph for allegedly not being described in the specification. Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Specifically, the Examiner asserts that the phrase "through intracellular processing" (added to the claims in the Reply dated June 8, 2001) is not found in the specification. Applicants respectfully disagree and submit that the term "processing" is well known in the art, especially in the field of MHC research.

Nevertheless, on page 5 of the Office Action, the Examiner states that amending the claims to recite "intracellular decomposition" will overcome the rejection. In the interest of expeditious prosecution and in order to overcome the rejection, Applicants amend the claims in accordance with the Examiner's suggestions.

Applicants respectfully submit that the instant claims are fully in compliance with 35 U.S.C. § 112, first paragraph. Withdrawal of the instant rejection is respectfully requested.

**Rejection under 35 U.S.C. § 112, second paragraph**

The Examiner rejects claims 6-9 and 12-13 under 35 U.S.C. §112, second paragraph for allegedly being indefinite. Applicants respectfully traverse. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

A. Claims 6-9 and 12-13 are rejected for the alleged recitation of "42°C,\_7" in claim 6. First, Applicants wish to clarify that claim 6, as submitted in the Reply dated June 8, 2001, recited "42°C,\_wherein." Second, the underline between the common and wherein of the phrase is simply a typographical error. Claim 6 is amended herein to properly recite "42°C, wherein". Thus, the instant rejection is overcome.

B. The Examiner points out that SEQ ID NO:1 is a nucleotide sequence whereas SEQ ID NO:2 is a amino acid sequence. As such, the descriptions of (a) and (b) in claim 6 are backwards. Applicants amend claim 6, as well as claim 8 and the abstract in view of the Examiner's remarks. Thus, the instant rejection is overcome.

C. The Examiner asserts that claims 7 and 12 are indefinite for reciting "consisting of part of the protein." In response to the Examiner's remarks, Applicants amend claims 7 and 12 to delete the term "part", and describe the peptide fragment(s) as the intracellular decomposition product of the claimed protein, as suggested by the Examiner above. Thus, the instant rejection is overcome.

Applicants respectfully submit that the above amendments render the instant claims in full compliance with 35 U.S.C. § 112, second paragraph. Thus, Applicants respectfully request that the instant rejection applied to the claims is withdrawn.

**Summary**

Overall, the present invention possesses significant patentable features that the cited prior art references do not possess. Furthermore, Applicants submit that amendments to the instant claims render them fully in compliance with 35 U.S.C. § 112, first and second paragraphs. All of the present claims define patentable subject matter such that this application should be placed into condition for allowance. Early and favorable action on the merits of the present application is thereby requested.

If there are any minor matters precluding allowance of the present application which may be resolved by a telephone discussion, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at (703) 205-8000.


Pursuant to 37 C.F.R. §§ 1.17 and 1.136(a), Applicant(s) respectfully petition(s) for a three (3) month extension of time for filing a reply in connection with the present application, and the

required fee of \$920.00 is attached to the Notice of Appeal, which is being filed concurrently herewith.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachment: Version with Markings to Show Changes Made  
Exhibit 1: T. Boon  
Exhibit 2: D. Hunt et al.

**ABSTRACT**

DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 2 or a variant protein thereof in which one or more amino acid residues are substituted, deleted or added, said protein or variant protein thereof being capable of yielding through its intracellular decomposition, peptide fragment(s) which can bind to major histocompatibility complex (MHC) class I antigen and which can be recognized by T cells in such binding state, medicines comprising said DNA as an active ingredient, expression plasmids comprising said DNA, transformants transformed with said expression plasmids, as well as tumor antigen proteins and tumor antigen peptides produced by expression of said DNA.

Version with Markings to Show Changes Made

IN THE ABSTRACT

The Abstract has been amended as follows:

DNA encoding a protein having the amino acid sequence shown in SEQ ID NO: 2 [SEQ ID NO: 1] or a variant protein thereof in which one or more amino acid residues are substituted, deleted or added, said protein or variant protein thereof being capable of yielding through its intracellular decomposition, peptide fragment(s) which can bind to major histocompatibility complex (MHC) class I antigen and which can be recognized by T cells in such binding state, medicines comprising said DNA as an active ingredient, expression plasmids comprising said DNA, transformants transformed with said expression plasmids, as well as tumor antigen proteins and tumor antigen peptides produced by expression of said DNA.

IN THE CLAIMS:

The claims have been amended as follows:

Claim 6 (Twice Amended)

An isolated tumor antigen protein selected from the group consisting of:

(a) a protein comprising an amino acid sequence shown in SEQ ID NO:2 [SEQ ID NO:1];

(b) a protein encoded by a DNA comprising a nucleotide sequence shown in SEQ ID NO:1 [SEQ ID NO:2]; and

(c) a protein of about 800 amino acids which is encoded by a DNA which hybridizes to a complement [compliment] of the DNA of SEQ ID NO:1 [SEQ ID NO:2] under stringent hybridization conditions comprising 6xSSC, 50% formamide, and 0.5% SDS and a temperature of 42°C,[\_wherein]

wherein said protein yields, through intracellular decomposition [processing], peptide fragment(s) which binds to major histocompatibility complex (MHC) class I antigen and is recognized by cytotoxic T lymphocytes (CTLs) in such binding state.

**Claim 7** (Twice Amended)

An isolated and purified tumor antigen peptide that is a peptide fragment of [consisting of part of] the protein of claim 6, which binds to MHC class I antigen and is [to be] recognized by CTLs when bound to MHC class I antigen.

**Claim 8** (Three Times Amended)

An isolated tumor antigen peptide of claim 7 which comprises the amino acid sequence of positions 749-757, 736-744, 785-793, or 690-698 in the amino acid sequence of SEQ ID NO:2 [SEQ ID NO:1].

# Teaching the Immune System To Fight Cancer

*Certain molecules on tumors can serve as targets for attack by cells of the immune system. These tumor-rejection antigens may provide a basis for precisely targeted anticancer therapy*

by Thierry Boon

At its best, the immune system is the ideal weapon against infectious disease. It eliminates viruses and bacteria that invade the body and kills infected cells, yet it leaves healthy tissue intact. The system is so precise because it responds only to specific targets called antigens: molecules or fragments of molecules that belong to the foreign invaders. In general, antibody molecules inactivate pathogens and toxins that circulate in body fluids, whereas white blood cells called cytolytic *T* lymphocytes destroy ("lyse") cells that have been penetrated by viruses.

The specificity and power of the immune system have not escaped notice of cancer researchers. Assuming that *T* lymphocytes might be able to eradicate cancer cells as effectively as they lyse virus-infected cells, investigators have long hoped to identify tumor-rejection antigens: structures that *T* lymphocytes can recognize on tumor cells in the body. These workers reasoned that antigens appearing exclusively (or almost exclusively) on cancer cells could be manipulated in ways that would trigger or amplify a patient's insufficient immune reaction to those targets.

Definitive evidence that tumor-rejec-

tion antigens exist on human tumors has been elusive. Yet in the past few years, my colleagues and I at the Ludwig Institute for Cancer Research in Brussels have gathered unequivocal proof that many, perhaps most, tumors do indeed display such antigens. Equally important, we have developed ways to isolate genes that specify the structure of these antigens. Moreover, we and others have seen indications that *T* lymphocytes that normally ignore existing tumor-rejection antigens can be prodded to respond to them. Hence, the design of therapies to generate such *T* cell responses to well-defined tumor-rejection antigens has finally become feasible.

The first clues that tumor-rejection antigens sometimes arise on tumors were uncovered in the 1950s, before the distinct roles of antibodies and *T* cells were elucidated. Several researchers—notably E. J. Foley of the Schering Corporation in Bloomfield, N.J., Richmond T. Prehn and Joan M. Main of the National Cancer Institute and George Klein of the Karolinska Institute Medical School in Stockholm—had generated cancers in mice by treating the animals with large doses of a carcinogenic compound. When the mice were freed of their tumors by surgery and subsequently injected with cells of the same tumor, they did not suffer a recurrence. The mice did acquire cancer after being injected with cells from other tumors, however. Those observations suggested that cells of carcinogen-induced tumors carry antigens that can elicit a response by the immune system.

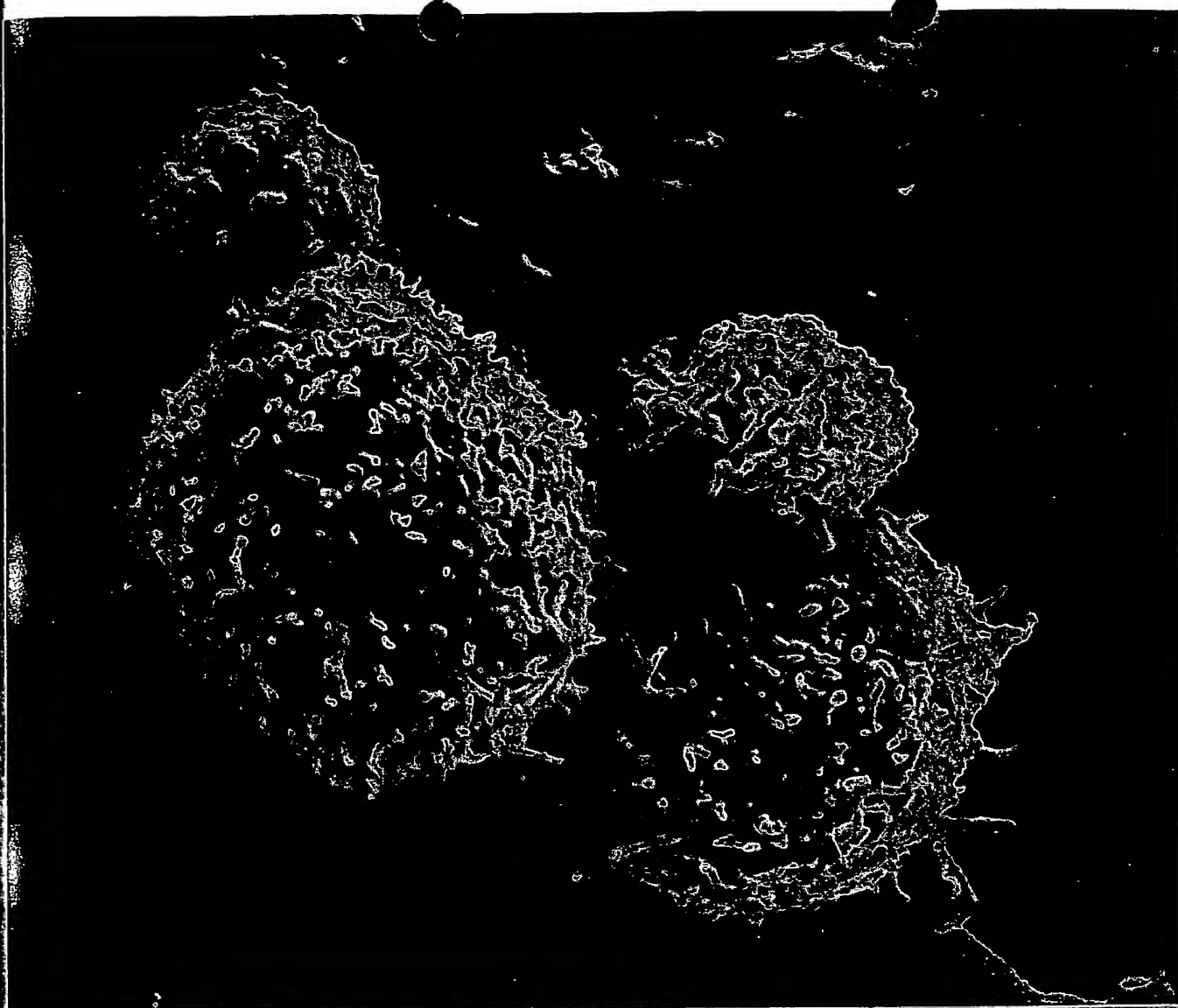
For about 20 years after those pioneering experiments were completed, hope ran high that human cancers, too, might bear tumor-rejection antigens. The prospect for antigen-based therapy

seemed even better when, toward the end of that period, *T* lymphocytes were found to be particularly important for ridding the body of abnormal cells. Jean-Charles Cerottini and K. Theodor Brunner of the Swiss Institute for Experimental Cancer Research in Lausanne showed that when mice reject tissue transplanted from an unrelated donor, the animals produce cytolytic *T* lymphocytes that can destroy cells from the transplant. By then it was apparent as well that when the specialized antigen receptors on cytolytic *T* lymphocytes bind to foreign antigens on a cell, the lymphocytes both lyse the cell and multiply, amplifying the immune reaction. These discoveries intimated that cancer researchers might make major strides if they concentrated on finding the antigenic targets of cytolytic *T* lymphocytes and on augmenting the activity of the cytolytic cells.

In the mid-1970s, however, experiments reported by Harold B. Hewitt, then at Mount Vernon Hospital in London, ushered in an era of pessimism. In contrast to the earlier experiments, which examined tumors induced by exposure to massive doses of carcinogens, Hewitt looked for evidence of tumor-rejection antigens on spontaneously arising malignancies. His careful work, conducted on many types of cancer, strongly suggested that spontaneous tumors in mammals did not evoke any immune rejection. Hence, he argued, the observations made in the earlier studies had little relevance to human tumors; people are rarely exposed to the high levels of carcinogens with which scientists produce malignancies in the laboratory.

Reasonably, many investigators then turned their attention elsewhere. Yet between 1972 and 1976 my colleagues and I had seen indications that tumor-rejection antigens were present on several mouse tumors that failed to elicit

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**WHITE BLOOD CELLS** called cytolytic *T* lymphocytes (*small spheres*) are attacking two cells from a mouse tumor called P815 (*large spheres*). Such lymphocytes bind to tumor cells when they recognize specific targets known as tumor-rejec-

tion antigens on the cell surface. Investigators have now found ways to identify the antigens with certainty. They hope to incorporate such antigens into therapies that will incite a patient's own *T* lymphocytes to eradicate tumors.

an immune rejection response. In addition, we discovered that the initially ineffective antigens could become useful targets for a defensive assault if the immune system were somehow made more aware of their existence. And so, even after Hewitt published his data, we remained hopeful that immunotherapies based on tumor-rejection antigens might be possible for humans.

**A**s often happens in science, we were studying a totally unrelated problem in 1972 when we stumbled onto those first clues. We were trying to identify genes that control the way cells in mammalian em-

bryos differentiate to become the specialized cells of mature organisms. My colleague Odile Kellermann and I, then at the Pasteur Institute in Paris, had exposed a culture of mouse tumor cells to a potent mutagen, a compound that introduces random, permanent changes (mutations) in genes. Then we put individual treated cells in separate plastic dishes and allowed them to proliferate so that each dish eventually contained a population of identical cells (a clone). That done, we transferred the clonal populations into mice and examined the cell types present in the tumors that resulted.

To our disappointment, the experi-

ments did not lead to a better understanding of the mechanisms of differentiation. But they did turn up a highly intriguing phenomenon. The original, or parent, tumor cells (those not yet exposed to the mutagen) almost always yielded cancerous growths when injected into mice. Yet many of the mutagen-treated clones produced no malignancies. Although I was a geneticist by training and knew little about cancer, I felt impelled to find out why the mutagen-treated cells did not form tumors. For simplicity's sake, my associates and I referred to cell clones that failed to generate tumors as *tum<sup>-</sup>* variants.

We found that the *tum<sup>-</sup>* variants

caused no cancer because the immune system of the injected mice had destroyed them, much as it might reject a mismatched kidney transplant. We found as well that the rejection occurred because the mutagen induced the tum<sup>-</sup> cells to display one or more antigens (tum<sup>-</sup> antigens) that elicited a potent *T* lymphocyte response; these tum<sup>-</sup> antigens were not present on the parental, tumor-inducing (tumorigenic) cell line and appeared to be different for every tum<sup>-</sup> variant.

The results were interesting by themselves, but what truly captivated us was a second finding I obtained with Aline Van Pel, after we joined the International Institute of Cellular and Molecular Pathology (ICP) in Brussels. As was true of the spontaneous cancers studied by Hewitt, the cells of our original tumor were totally incapable of eliciting an immune attack. Yet often when we injected these cells into mice that had rejected one or another tum<sup>-</sup> variant, no cancer developed. In mounting an immune response to a tum<sup>-</sup> variant, the mice somehow acquired resistance to the original tumor cell. The mice did not resist unrelated cancers, however, indicating that rejection of the original tumor cells was caused by an antigen shared by the tum<sup>-</sup> variant and its parent but not by other cancers.

Our findings were later confirmed in several follow-up studies involving many different mouse tumors. Most important, Van Pel observed that she could reproduce our results with the very spontaneous tumors Hewitt had examined. Clearly, the conclusion that spontaneous cancers did not display tumor-rejection antigens had to be revised.

No one has fully explained how tum<sup>-</sup> variants manage to induce a powerful immune response to the initially ineffectual, or weak, antigens on the original cells. We suspect that small proteins called interleukins play a role. A lymphocyte that has bound to an antigen releases interleukins. These proteins, in turn, promote proliferation of that lymphocyte and nearby ones (such as those bound to another antigen on the same tumor cell or on neighboring cells). It seems probable that the tum<sup>-</sup> antigens are potent enough to spur *T* lymphocytes to kill tum<sup>-</sup> cells and to multiply rapidly even in the absence of preexisting interleukins in the local environment. These lymphocytes then produce interleukins, which help other *T* cells become activated by weak tumor-rejection antigens. Consistent with this view is the fact that in recent years several research groups have modified tumor cells to secrete interleukins. In many instances, the workers have seen a con-

siderable increase in the immune response to the tumors.

By the early 1980s, then, our collected evidence suggested the following conclusion: mouse tumors that normally fail to elicit a buildup of *T* lymphocytes nonetheless often carry weak antigens that can become targets for an effective immune assault. Because the immune system of mice is much like that of humans, the data implied that human tumors might be antigenic as well. If so, they might be susceptible to immunotherapy that artificially induced an antigen-specific attack. In other words, immunotherapy for humans was a reasonable goal. At that point, we decided to apply all the forces of our laboratory to the study of tumor-rejection antigens.

**B**efore considering therapy, we would have to identify specific tumor-rejection antigens. All earlier attempts to isolate such structures directly from cell membranes in human and mouse tumors had failed. We therefore decided to try an alternative approach: cloning, or isolating, the genes that direct construction of the antigens. Unfortunately, no one had yet come up with a good way to perform the task. And so in 1983 my colleagues and I, by then members of the Ludwig Institute, set out to develop a method of our own. It took us four years to devise an approach that would work in a test system [see box on opposite page].

In our first successful cloning effort, we isolated the gene for the tum<sup>-</sup> antigen appearing on the cells of a mouse tum<sup>-</sup> variant. Of course, tum<sup>-</sup> antigens are not true tumor-rejection antigens, because they are artificially induced to appear on cultured tumor cells and are not found on cancers in the body. But, as will be seen, they were useful for our trial run. We generated the tum<sup>-</sup> variant from a cell line that was derived from a mastocytoma (mast cell tumor) named P815. The original P815 cell line was appealing for our purposes because the cells replicate rapidly and indefinitely in the test tube. In addition, tum<sup>-</sup> variants of P815 cells provoke cytolytic *T* lymphocytes into a strong, readily detectable response.

Our gene-cloning plan relied first of all on having a good supply of cytolytic *T* cells reactive to the tum<sup>-</sup> antigen of the variant. The *T* cells would later lead us to the gene for the antigen. To acquire the cytolytic cells, we injected the P815 tum<sup>-</sup> variant into mice. Then we removed the spleen (a repository of lymphocytes) from animals that rejected the variant. We knew that if the lymphocytes from these immunized animals were exposed to killed cells of the

variant, cytolytic *T* lymphocytes specific for the variant would multiply preferentially; other lymphocytes would disappear. (Tumor cells would be killed to prevent them from overtaking the culture.) When this culturing was done, we had a supply of cytolytic *T* lymphocytes of which some responded to the tum<sup>-</sup> antigen and others to tumor-rejection antigens present on all P815 cells. By placing individual lymphocytes in laboratory dishes and allowing them to replicate separately, we obtained several clones that would lyse only the tum<sup>-</sup> variant and could be made to multiply indefinitely in laboratory dishes. We chose one of the clones directed against the tum<sup>-</sup> antigen to use in the quest for the gene.

In outline, the plan for isolating the gene for the tum<sup>-</sup> antigen was straightforward. We intended to collect all the genetic material of the variant. Next we would link fragments of this DNA to pieces of bacterial DNA, which would later serve as labels to help retrieve the gene of interest. We would introduce the fragments into cells that do not normally produce the tum<sup>-</sup> antigen. Then we would test the ability of each of these cells to stimulate our *T* lymphocyte clone. We would know that a recipient cell displayed the antigen (and thus had taken up the corresponding gene) if the cell spurred the lymphocytes to proliferate. By searching for the bacterial label we had attached to the DNA of the tum<sup>-</sup> variant, we would locate and retrieve from the DNA of the recipient cell the gene for the tum<sup>-</sup> antigen.

Although the plan was relatively simple conceptually, the implementation was quite laborious. Mammalian cells contain approximately 100,000 distinct genes, spread throughout roughly three billion nucleotides (the building blocks of DNA) in the chromosomes inherited from each parent. Because of inefficiencies in the techniques available for inserting DNA into recipient cells, we had to create a gene "library" containing millions of copies of each gene. These copies were obtained by splicing fragments of the DNA from the tum<sup>-</sup> variant into 300,000 plasmids, or circular bits of bacterial DNA; each such plasmid carried about 40,000 nucleotides of inserted tum<sup>-</sup> DNA (containing an average of one or two genes). After allowing the plasmids to multiply in bacteria, we recovered the DNA.

Next we selected as the recipient a cell type that could incorporate such plasmids into its chromosomes. The original P815 line proved suitable. To ensure that at least one copy of each gene in the tum<sup>-</sup> variant would fit into the DNA of the recipient P815 cells, we

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had to mix the recovered plasmids with more than 300 million P815 cells. We needed that many because we knew only about one in 10,000 of the cells would take up DNA. We also knew that these few cells would accept a lot of DNA—500,000 nucleotides on average.

Fortunately, we were able to avoid having to test every last cell for its ability to activate the selected clone of T lymphocytes. We did so by including in the bacterial DNA a gene that conferred resistance to a particular toxic drug. When we treated the full set of cells with the drug, we eliminated all those that had failed to integrate a plasmid into their DNA. We were thus left to test just 30,000 of the original 300 million P815 cells. By testing small groups of the 30,000 cells, we found the few that stimulated the T lymphocytes to multiply. We then homed in on the bacterial DNA of one of these cells and thus picked out the tum<sup>-</sup> DNA. By repeating much the same process with this DNA fragment, we were soon able to isolate the gene giving rise to the tum<sup>-</sup> antigen.

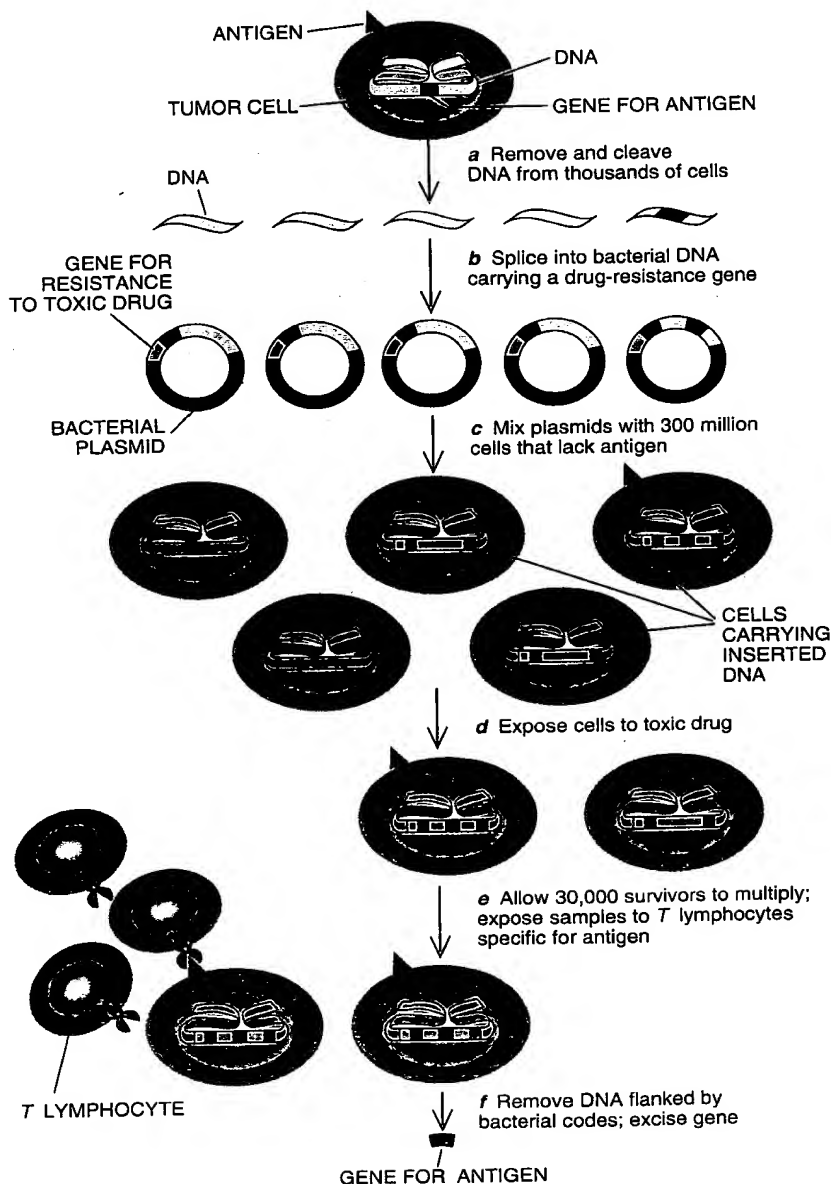
**W**e quickly deciphered the sequence of nucleotides in the gene. The sequence did not resemble that of any gene known at the time. We did find, however, that the gene was expressed not only in the tum<sup>-</sup> variant but also in the original P815 cells and in normal mouse tissue. That is, the gene, which specifies the sequence of amino acids to be strung together into a protein, was being transcribed into molecules of messenger RNA that were, in turn, being translated into protein.

Expression in normal cells meant that our gene specified a standard component of cells. But all was not normal in the tum<sup>-</sup> variant. There the gene had suffered a point mutation, causing one amino acid to be substituted for another in the protein product. The same was true of two other tum<sup>-</sup> genes we cloned later. We were puzzled. How could a single amino acid substitution transform a constituent of normal cells into a strong antigen recognized by cytolytic T lymphocytes?

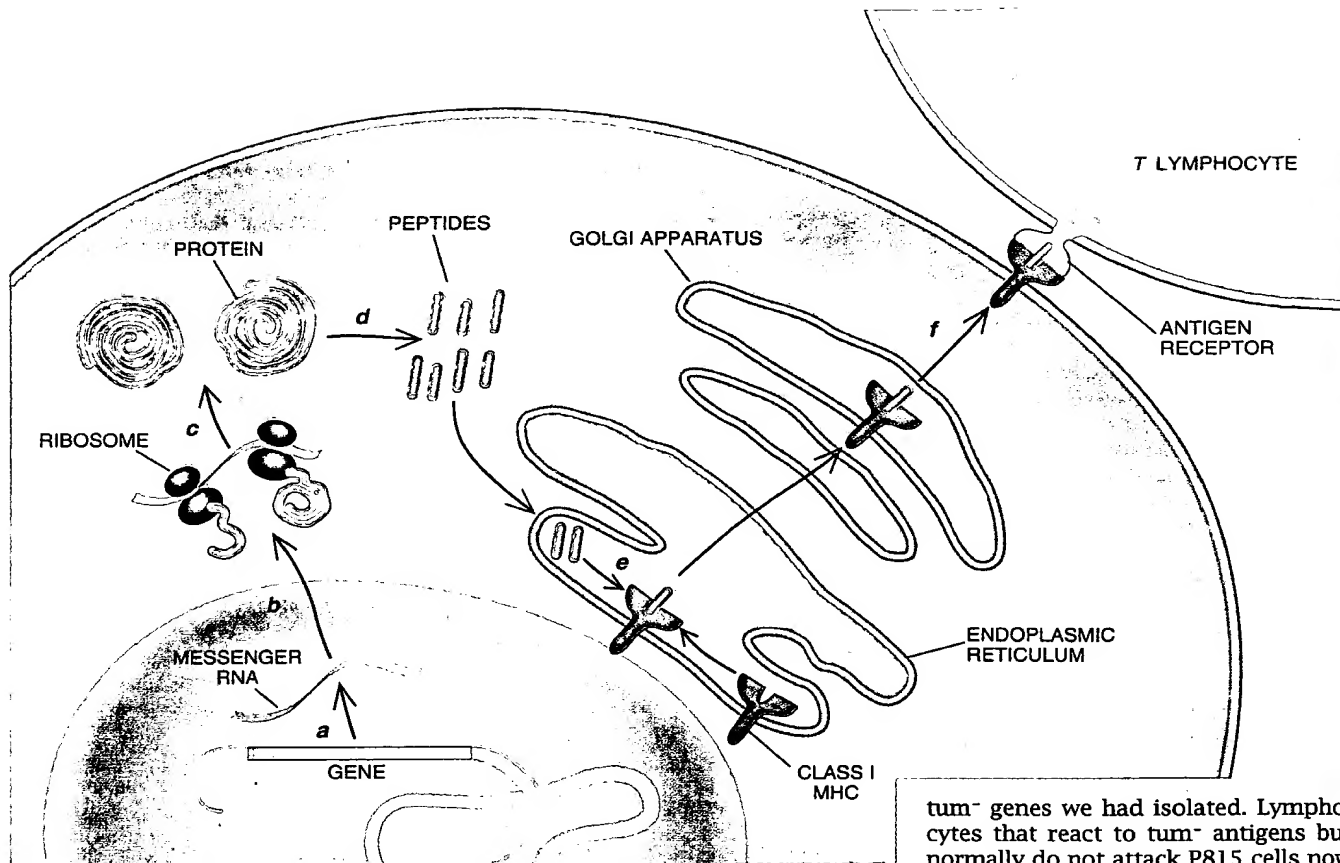
Just when we were asking this question, Alain R. M. Townsend of John Radcliffe Hospital in Oxford, England, and his colleagues made a discovery that led us to the answer. In 1986 they demonstrated that cytolytic T lymphocytes can often detect viral proteins hidden within cells. In contrast, antibodies respond only to proteins that exert their functions on the cell surface. The T cells can accomplish this feat because, in the course of mammalian evolution,

## How Genes for Antigens Recognized by T Lymphocytes Are Cloned

**C**loning, or isolation, of a gene (red band in nucleus) for an antigen (red triangle) on a tumor cell begins with removal and cleavage of DNA from multiple copies of the cell (a). Workers insert the resulting DNA fragments into plasmids (rings of bacterial DNA) bearing a gene (yellow) that confers resistance to a toxic drug (b). They mix the plasmids with cells that lack the antigen, causing some of those cells to take up one or more plasmids (c). Next investigators expose the cells to the toxic drug (d), thereby eliminating any cells that have failed to incorporate the plasmid DNA into their own DNA. The surviving cells are allowed to multiply, and samples are exposed to T lymphocytes that specifically recognize the antigen of interest (e). Any cell that induces a lymphocyte response (such as proliferation) can be assumed to produce the antigen, which means it also harbors the corresponding gene. Hence, researchers remove the foreign DNA from an identical cell, excise the bacterial DNA and fish out the desired antigen-specifying gene (f).



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**CELLS PRODUCE ANTIGENS (red and green complex at top right) in a multistep process. Once a gene (red band at bottom) directs synthesis of a protein (a-c), cellular enzymes chop these proteins (large red coils) into fragments (small red bars) called peptides (d). Some of these peptides are then transported into an intracellular compartment (the endoplasmic reticulum) (e), where they may combine with so-called class I major histocompatibility (MHC) molecules (green). Such peptide-MHC complexes are transported to the cell surface (f), where *T* lymphocytes (orange body at top right) can examine them.**

tum<sup>+</sup> genes we had isolated. Lymphocytes that react to tum<sup>+</sup> antigens but normally do not attack P815 cells now lysed the cells. But the lymphocytes did not lyse P815 cells that were mixed with peptides encoded by the normal sequences of the genes. Later we showed that the point mutations in two of the tum<sup>+</sup> genes had rendered the affected peptides capable of binding to MHC molecules. The normal versions of these peptides do not bind and consequently are never displayed to the immune system. For the third mutated gene, the situation was different. The normal version of the altered peptide does in fact bind to MHC molecules. But because it is a constituent of the self, the process of natural tolerance had eliminated any *T* lymphocytes responsive to it. The mutation changed the shape of the exposed part of the peptide so that the peptide could now be detected by an existing *T* cell population.

an elaborate protein-surveillance system has arisen. Cellular enzymes routinely chop a fraction of all the proteins in the cytoplasm into small fragments known as peptides. These peptides are transported to a special intracellular compartment, the endoplasmic reticulum. There some of them fit themselves into a groove within specialized proteins known as class I major histocompatibility (MHC) molecules. (In humans, MHC molecules are also called human leukocyte antigens, or HLA molecules.) The MHC-peptide complexes move to the surface and become anchored in the cell membrane, ready to be scrutinized by cytolytic *T* cells. Lymphocytes whose antigen receptors can bind to such a complex may then attack the cell. Thus, peptides derived from normal proteins are continuously displayed. This presentation does no harm because of natural tolerance: early in life the body eliminates all *T* lymphocytes that recognize the constituents of the self. But if a peptide is derived from a foreign protein, such as

that of a virus hiding within a cell, then a *T* lymphocyte will notice it and attempt to kill the cell.

**O**n the basis of these discoveries, we surmised that the point mutations in the three tum<sup>+</sup> genes had converted peptides that were not seen by *T* lymphocytes to ones that were seen. To test this idea, we made use of a crucial observation of Townsend and his colleagues. They had found that healthy cells could be rendered instantly recognizable to antiviral cytolytic *T* lymphocytes if the cells were put in a medium containing a synthetic version of a small peptide belonging to a viral protein. Presumably, the healthy cells stimulated the lymphocytes because a few MHC molecules on the surface had taken up the peptides and presented them to the *T* cells.

We conducted similar experiments to reveal the role of tum<sup>+</sup> mutations. We mixed P815 cells with small peptides (of nine to 10 amino acids) coded for by the mutated regions of the three

Conceivably, a mutation in virtually any gene can result in the appearance of a new antigen on a cell. Accordingly, an infinite variety of antigens can be produced by random mutations. The diverse antigens that appear on rodent tumors induced by chemical carcinogens probably arise through such a mechanism. In addition, mutations can occasionally transform normal genes into ones that cause cancer (oncogenes). Some of these oncogenic changes may well generate antigenic peptides that will one day serve as targets for specific immunotherapy.

Having demonstrated the merit of our

cloning technique, we set about isolating a gene of a bona fide tumor-rejection antigen—one present on a cancer that grows in an animal. Fortunately, we had at our disposal a cytolytic *T* lymphocyte clone that lysed the original P815 cells and did not lyse normal mouse cells. Clearly, the gene specifying the tumor-rejection antigen (named P815A) recognized by these lymphocytes was a logical target for our gene search.

Before starting, however, we wanted to be sure this antigen—which was identified by cytolytic *T* lymphocytes in the test tube—could also direct an immune response to a tumor in the body. We were able to address this question because we had observed an odd effect of P815 cells. Usually when mice are injected with those cells, tumors appear within a month. Yet a few mice formed tumors only after a long delay. When malignancies finally emerged, they resisted attack by the cytolytic *T* lymphocytes responsive to P815A. We concluded, correctly as it turns out, that these animals had rejected almost all the P815 cancer cells because, in the body, *T* lymphocytes identical to those in the clone had recognized antigen P815A. But a few tumor cells had stopped displaying P815A because they had lost

the gene specifying it. These so-called antigen-loss variants had proliferated, accounting for the eventual tumor formation. This work demonstrated that an antigen recognized by cytolytic *T* lymphocytes in a laboratory dish might also be of value for eliciting a tumor-rejection response in the body.

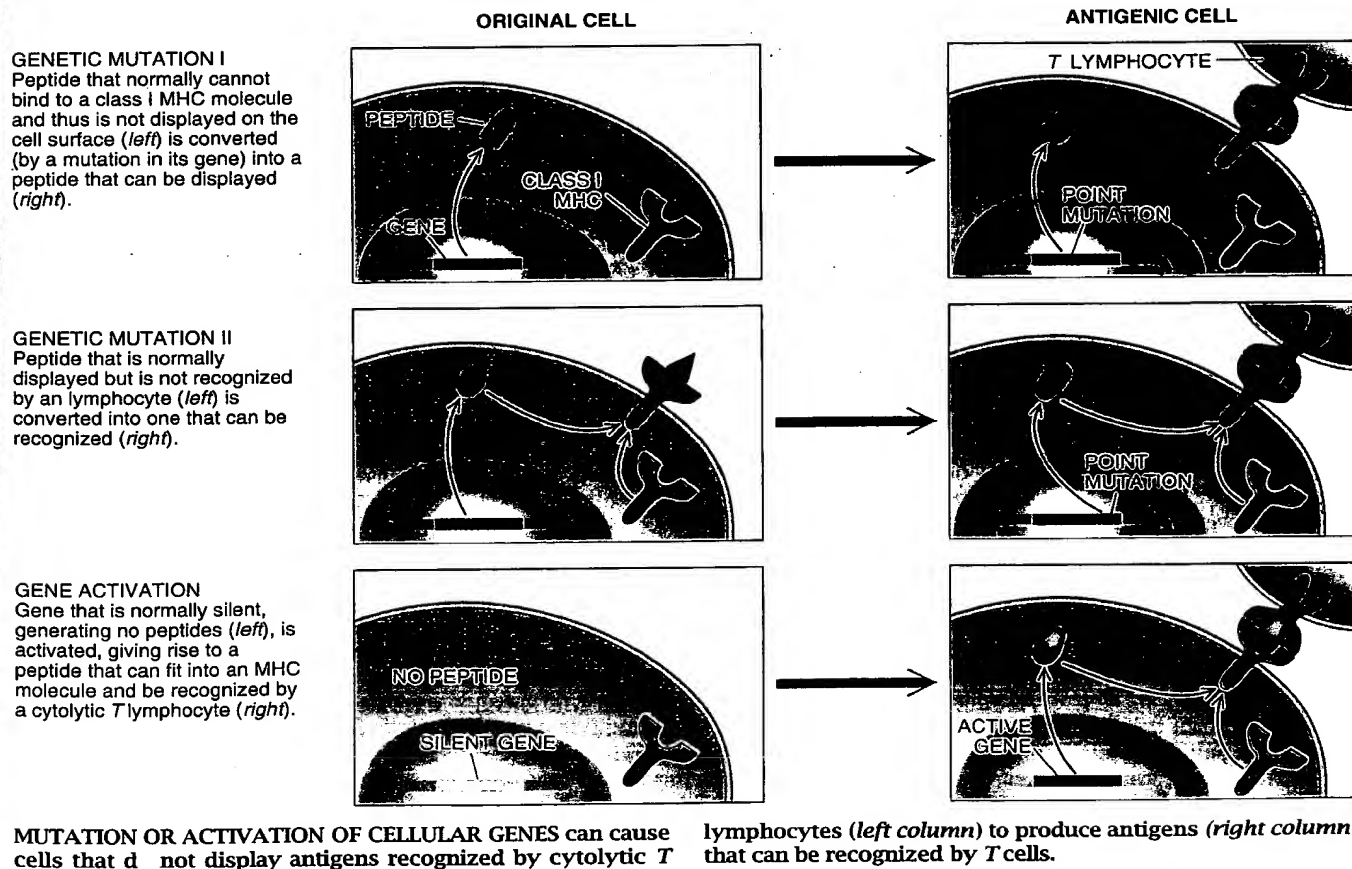
Conveniently, such antigen-loss variants could be used as DNA recipients in our efforts to clone the gene coding for antigen P815A. We isolated the gene by applying our by then well-tuned cloning procedure. We built a gene library with DNA from P815 cells and transferred this DNA into cells of an antigen-loss variant. We then fished out the gene from one of the few recipients that incited proliferation of our *T* lymphocytes responsive to antigen P815A. We named the gene *P1A*.

The nucleotide sequence of the *P1A* gene was found to be identical in P815 cells and in normal mouse cells. But in normal cells the gene is inactive; it produces no protein and therefore no antigenic peptide. P815 tumors express the gene and thereby generate an antigen that does not appear on normal cells. Thus, expression of usually silent genes is yet another mechanism of antigen formation. We expected that this last mech-

anism would generate antigens common to tumors of many different individuals. After all, probably only a relatively limited set of genes can help cancer cells multiply and spread throughout the body. Therefore, we were not surprised to observe that several mastocytoma tumor cell lines express the *P1A* gene, whereas normal mast cells do not.

By 1989 we were ready to begin searching for genes encoding tumor-rejection antigens on human cells. We focused on a cell line named MZ2-MEL, derived from a melanoma tumor (a form of skin cancer) that had formed in a 35-year-old woman known as patient MZ2. We isolated a gene on the cell line in much the same way we obtained the mouse *P1A* gene.

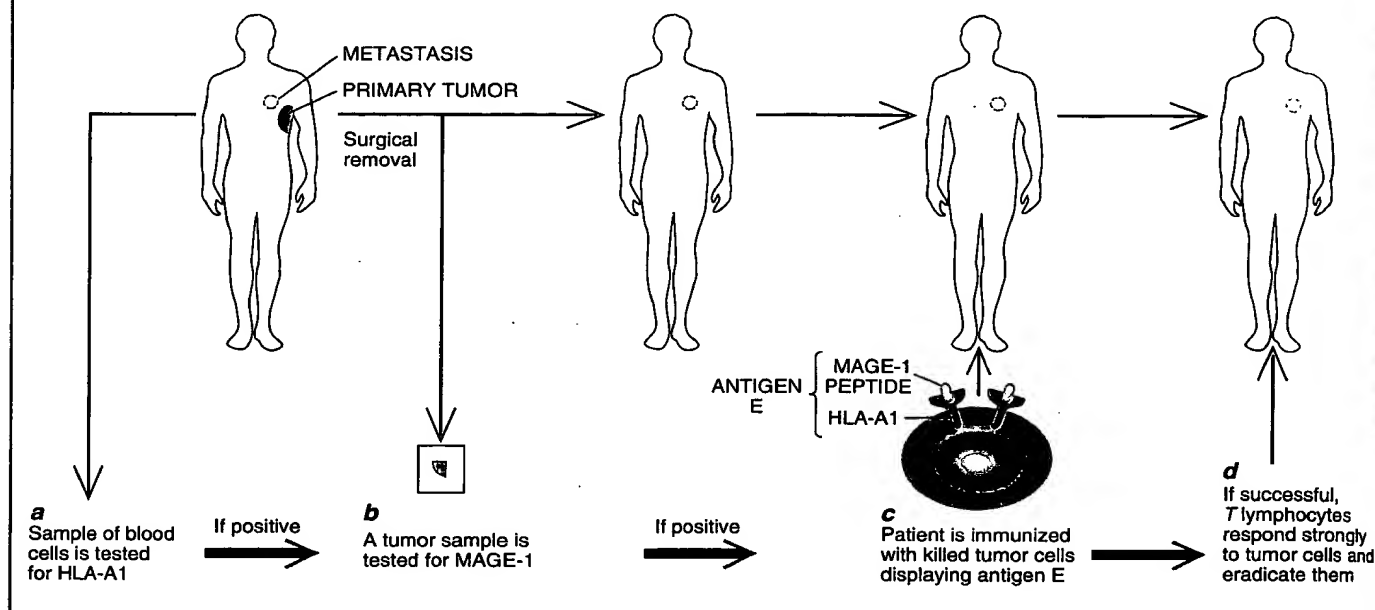
As a first step, we isolated from the patient's white blood cells cytolytic *T* lymphocytes that reacted to the MZ2-MEL cells. Like several other groups working with other tumors, we managed to garner such lymphocytes by culturing the patient's white cells with killed cells from her tumor. Although the original tumor failed to induce rejection in the body, culturing the cells for a few weeks enabled us to isolate cy-



## Scheme for Specific Immunotherapy

One immunotherapy now being considered is based on the discovery that cytolytic *T* lymphocytes isolated from some cancer patients can be induced to react to a molecular complex called antigen E. Antigen E is formed by a specific MHC molecule (called HLA-A1) and a peptide derived from a protein called MAGE-1. Melanoma patients whose

cells produce the HLA-A1 molecule (a) and whose tumors additionally produce the MAGE-1 protein (b) will be injected with killed cells displaying antigen E (c). If all goes well (d), *T* lymphocytes specific for antigen E will proliferate markedly and eradicate tumors. The diagram at the far right represents screening results obtained by the polymerase chain reaction,



tolytic *T* lymphocytes that selectively lysed the tumor cells. From this potentially mixed population of antitumor lymphocytes, we generated clonal populations that were each reactive to a single antigen.

We also needed an antigen-loss variant that could serve as the recipient for DNA from MZ2-MEL cells. This time we obtained the variant by exposing several million MZ2-MEL cells to a similar number of lymphocytes from one cytolytic *T* cell clone—called the anti-E clone because its target antigen was named (arbitrarily) “E.” Most of the tumor cells died, but about one in a million lived. These survivors turned out to have lost antigen E. The antigen-loss variants proved sensitive to other *T* cell clones directed against MZ2-MEL cells. Eventually this finding led to the discovery that the MZ2-MEL tumor displays at least four distinct tumor-rejection antigens.

So far we have isolated only the gene that gives rise to antigen E. As might be expected from the *PIA* work, we did so by inserting plasmids carrying the DNA of MZ2-MEL cells into cells of a variant that had lost antigen E. Then we withdrew the gene from one of the few antigen-loss cells that activated the

anti-E lymphocyte clone. We named the gene *MAGE-1*, for *melanoma antigen-1*.

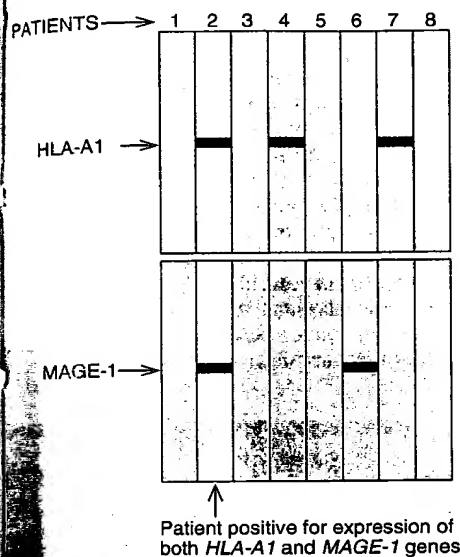
As soon as we knew the nucleotide sequence of this gene, we rushed to determine whether normal cells of the patient carried the sequence. They did, but the gene was not expressed. Here again a tumor-rejection antigen had arisen through the activation of a gene that is silent in normal cells. This finding intimated that, in analogy with *PIA*, the gene might be active in tumors of other patients as well. Indeed, analyses of a large selection of tumor samples suggest that more than 30 percent of melanomas carry an active form of the *MAGE-1* gene. More than 15 percent of breast and lung tumors also express the gene. We have not yet discerned how the *MAGE-1* protein promotes tumor progression.

Do these figures mean that all patients who express the *MAGE-1* gene also display antigen E on tumor cells? The answer is no, for reasons that have to do with how antigens form. Recall that the *T* cell receptor actually recognizes not a solitary peptide but a complex consisting of a peptide and the surrounding region of the class I MHC molecule. Now, human class I molecules are encoded by three genes (named *HLA-A*,

*-B* and *-C*), and these genes are polymorphic; that is, they can differ from one person to another. Each gene, in fact, comes in 10 to 40 different forms, called alleles. Because a person inherits one set of A, B and C alleles from the mother and another set from the father, an individual can manufacture six different varieties of HLA proteins—such as *HLA-A1*, *-A10*, *-B7*, *-B24*, *-C4* and *-C6*—all of which might differ from the six varieties produced by someone else. The protein products of the alleles differ from one another in the shape of the peptide-binding groove and of the surrounding region. Consequently, in any given cell, a peptide typically binds to only one of the available class I molecules, if it binds at all. Hence, only patients who produce the *MAGE-1* protein and a particular HLA molecule will display antigen E. We now know the MHC component of antigen E is *HLA-A1*. We have also found that the *MAGE-1* peptide that binds to this HLA molecule is nine amino acids long, and we know its sequence.

Might patients who lack *HLA-A1* but produce the *MAGE-1* protein also display antigens that can be recognized by *T* lymphocytes? At this point, we do not know. In theory, such antigens

a test that can detect expression of the genes giving rise to the HLA-A1 and MAGE-1 proteins. Of eight patients tested, three expressed the *HLA-A1* gene, and two bore tumors that express the *MAGE-1* gene. Only one individual (patient 2) had positive test results in both categories.



could be created if peptides belonging to the MAGE-1 protein were capable of binding to HLA molecules other than HLA-A1. But we cannot be certain that such antigens exist until we identify cytolytic T lymphocytes that react to them. So far we have been unable to obtain such lymphocytes. The T cells that recognize antigen E would not respond to those antigens because they bind only to the specific shape formed by the peptide in antigen E and the part of the HLA-A1 molecule that surrounds it.

The identification of the gene coding for a human tumor-rejection antigen opens a new phase in the search for an effective specific immunotherapy for cancer. For the first time, we can select as candidates for therapy those patients who have a chance of benefiting from immunization. We can be selective because it is possible to readily identify individual patients whose tumors carry the known antigen. Further, having the gene for a tumor-rejection antigen means we can devise many innovative ways to immunize patients. Finally, we also have the opportunity to determine rapidly whether the immune system is responding to our interventions, because we can measure

changes in the number of a patient's cytolytic T lymphocytes instead of waiting until clinical effects become apparent (such as the absence of relapse).

We are now initiating clinical studies designed to immunize melanoma patients against antigen E. In these initial studies, we will concentrate on evaluating the cytolytic T cell response to the antigen. If we find reliable ways to elicit a good response, later trials will examine cancer remission.

Our methods of identifying candidates for therapy are simpler than might be imagined. We just need to know that their tumors express both the *HLA-A1* and the *MAGE-1* genes. Patients who are about to undergo surgery to remove a tumor can be tested for their HLA type in a couple of ways. One of these methods, based on a small sample of blood, yields results in a few hours. In individuals who test positive for HLA-A1, a sample of tumor can be frozen immediately after surgery. Within two days, a sophisticated technique called the polymerase chain reaction will reveal whether the tumors also express the *MAGE-1* gene [see "The Unusual Origin of the Polymerase Chain Reaction," by Kary B. Mullis; *SCIENTIFIC AMERICAN*, April 1990]. About 26 percent of white individuals and 17 percent of black individuals carry the *HLA-A1* allele. Considering that some 30 percent of melanoma patients express the *MAGE-1* gene, we can predict that roughly 8 percent of melanoma patients will display antigen E on their tumor cells.

A number of immunization modes can be tested on candidates who fit our dual criteria. Because the *MAGE-1* gene and the antigenic peptide have been identified, we can induce various cell types to express antigen E. Killed versions of the cells can be injected into patients to spur their anti-E lymphocytes into action. Our first clinical studies will follow such a protocol.

We also hope to evaluate the effectiveness of inserting a gene for an interleukin, such as interleukin-2, into cells expressing antigen E. The interleukin should facilitate the activation of T lymphocytes around these cells. Synthetic E peptides or purified MAGE-1 proteins that have been mixed with an immune stimulatory substance called an adjuvant will also be tried. Finally, we might insert the *MAGE-1* gene into the DNA of a harmless virus that can penetrate into human cells but cannot reproduce there. After such recombinant viruses are administered to patients, a relatively small number of cells should become infected. These cells should produce the MAGE-1 protein and display antigen E for a while. Immuniza-

tion with peptides, proteins and recombinant viruses has already proved quite effective for other purposes.

I do not know whether these treatments will cure patients, but I believe there is a good chance that some form of specific immunotherapy will be helpful. My associates and I are encouraged by mouse studies in which strong anti-tumor responses have been obtained without hurting the general health of the animals. But it is difficult to predict whether the specific immunotherapies I have described will eradicate human cancers, particularly in patients who harbor large tumors. Malignant cells that have lost the ability to produce the MAGE-1 or HLA-A1 protein may arise. Such cells would no longer make antigen E and would thus escape notice of the anti-E lymphocytes. Success, then, may have to wait until we can immunize cancer patients with several tumor-rejection antigens simultaneously. These multiple immunizations should strengthen the immune reaction and also help to prevent variants that have lost one antigen from escaping destruction.

We are confident that the gene-cloning techniques we have developed will lead in the near future to identification of additional genes specifying tumor-rejection antigens. The advances will make it feasible to attack tumors through several antigens. And they will render increasing numbers of patients eligible for trials of specific immunotherapies. Thus, even though success is by no means assured and the work ahead remains considerable, a clear strategy has now been mapped out for the specific immunotherapy of cancer.

#### FURTHER READING

THE EPITOPES OF INFLUENZA NUCLEOPROTEIN RECOGNIZED BY CYTOTOXIC T LYMPHOCYTES CAN BE DEFINED WITH SHORT SYNTHETIC PEPTIDES. A. R. M. Townsend, J. Rothbard, F. M. Gotch, G. Bahadur, D. Wraith and A. J. McMichael in *Cell*, Vol. 44, No. 6, pages 959-968; March 28, 1986.

A GENE ENCODING AN ANTIGEN RECOGNIZED BY CYTOLYTIC T LYMPHOCYTES ON A HUMAN MELANOMA. P. van der Bruggen, C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth and T. Boon in *Science*, Vol. 254, pages 1643-1647; December 13, 1991.

A MOLECULAR MODEL OF MHC CLASS-I-RESTRICTED ANTIGEN PROCESSING. John J. Monaco in *Immunology Today*, Vol. 13, No. 5, pages 173-179; May 1992.

TOWARD A GENETIC ANALYSIS OF TUMOR-REJECTION ANTIGENS. Thierry Boon in *Advances in Cancer Research*, Vol. 58, pages 179-210; 1992.

# Characterization of Peptides Bound to the Class I MHC Molecule HLA-A2.1 by Mass Spectrometry

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Antigens recognized by T cells are expressed as peptides bound to major histocompatibility complex (MHC) molecules. Microcapillary high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry was used to fractionate and sequence subpicomolar amounts of peptides isolated from the MHC molecule HLA-A2.1. Of 200 different species quantitated, eight were sequenced and four were found in cellular proteins. All were nine residues long and shared a distinct structural motif. The sensitivity and speed of this approach should enhance the analysis of peptides from small quantities of virally infected and transformed cells as well as those associated with autoimmune disease states.

CYTOTOXIC T LYMPHOCYTES (CTLs) are a part of the immune system concerned with recognition of host cells that express new antigens as a result of viral infection or transformation. CTLs do not recognize new antigens directly, but only as short peptides bound to a deep cleft in class I molecules of the MHC (1-3). Newly synthesized viral and cellular proteins are degraded into peptides in the cytoplasm, transported to the endoplasmic reticulum where they bind to class I molecules, and then expressed on the cell surface (4-7). Each of the allelic forms of the class I MHC molecule binds to a complex mixture of structurally distinct peptides (8, 9). Information on the nature of these peptides has been obtained from studies with synthetic peptides (10-12) and from Edman degradation applied to unfractionated mixtures of peptides extracted from five different class I MHC molecules (8). Sequences of 11 peptides extracted from HLA-B27 were identified after high-performance liquid chromatography (HPLC) fractionation and Edman degradation (9). Because HPLC was unable to completely resolve the complex mixture, this analysis could only be applied to the few fractions that contained one or two dominant peptides. Declining PTH (phenylthiohydantoin)-amino acid yields made it difficult to determine the exact number of residues in several peptides.

We have applied microcapillary HPLC-

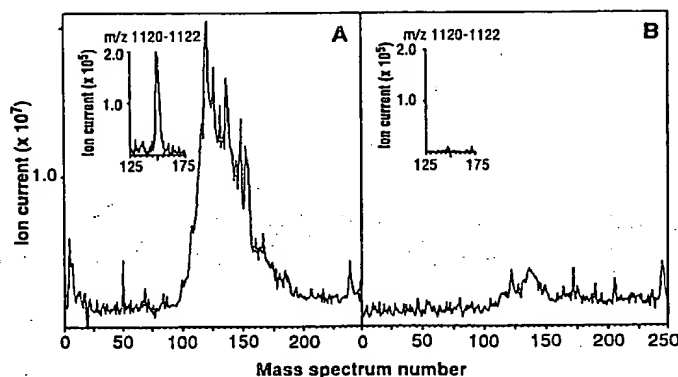
electrospray ionization-tandem mass spectrometry to circumvent the above problems. In a matter of hours, this technique determines the molecular mass and therefore maximum length of each peptide component, and the approximate number and quantity of individual peptides. Sequence information can be also obtained on subpicomolar amounts of peptides. We analyzed the naturally processed peptides bound to HLA-A2.1, one of the most widely distributed class I molecules within the human population. The three-dimensional structure of this molecule allows modeling of the complex (2).

HLA-A2.1 molecules were purified by immunoprecipitation from the human B lymphoblastoid cell line C1R-A2.1. The associated peptides were released by acid extraction and separated from HLA-A2.1 and

antibody by filtration. A "mock" extract was prepared from an equal number of Hmy2.C1R (C1R) cells, which do not express any HLA-A or -B products (13). Since C1R-A2.1 is a transfectant of C1R, these cell lines should be identical except for the expression of HLA-A2.1. Extracts were fractionated by microcapillary reversed-phase HPLC and eluted directly into the electrospray ion source of a triple quadrupole mass spectrometer (14). A broad band of material was observed in the extract from C1R-A2.1 (Fig. 1A), but not in the extract from C1R (Fig. 1B). If all HLA-A2.1 molecules contain bound peptide, the total peptide yield from  $10^8$  C1R-A2.1 cells should be roughly 20 pmol; we estimate that our total yield of peptide was about 70 to 80% of the amount expected. This provides evidence that the material isolated for C1R-A2.1 cells by this method is specifically associated with the HLA-A2.1 molecule.

Material with a nominal mass-to-charge ratio ( $m/z$ ) of 1121 was present in the extract from C1R-A2.1 cells but not that from C1R cells (Fig. 1 insets). By a similar analysis, approximately 200 distinct ions having a signal-to-noise ratio ( $S/N$ ) of at least 2 were observed in the material eluted from C1R-A2.1 cells. Of these, 47% were singly charged, 45% were doubly charged, and 8% were triply charged. Calculated molecular masses ranged between 737 and 1370. Known quantities of synthetic peptides added to cell extracts allowed quantitation of peptides, and defined the lower limit of detection ( $S/N = 2$ ) as 30 fmol. Of the 200 peptides detected, 10% were present at 150 to 600 fmol per  $10^8$  cells,

Fig. 1. Total ion chromatograms of HLA-A2.1-associated peptides. C1R-A2.1 (A) or C1R (B) cells ( $2 \times 10^9$ ) were solubilized in 20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40, 2 mM PMSF, 100  $\mu$ M iodoacetamide, Aprotinin (5  $\mu$ g/ml), Leupeptin (10  $\mu$ g/ml), Pepstatin A (10  $\mu$ g/ml), EDTA (3 ng/ml), and 0.2% sodium azide. After sample centrifugation at 100,000g for 1 hour, supernatants were immunoprecipitated with the HLA-A2.1-specific mono-



clonal antibody BB7.2 (30) and protein A-Sepharose (Sigma). Precipitates were extracted with 0.2 N acetic acid. The supernatants were brought to pH 2.1 with glacial acetic acid and boiled for 5 min, centrifuged through an Ultrafree-C1 unit (Millipore) with a nominal limit of 5 kD, and concentrated by vacuum centrifugation. Mass spectra were recorded on a Finnigan-MAT TSQ-70 (San Jose, California) triple quadrupole mass spectrometer equipped with an electrospray ion source and a C-18 microcapillary-HPLC column (75  $\mu$ m by 10 cm) (14). The column was eluted with a gradient of 0 to 80% acetonitrile in 0.5% acetic acid over 10 min at 1 to 2  $\mu$ l/min. The electrospray needle was operated with a voltage differential of 4 to 5 kV and a sheath flow of 2 to 4  $\mu$ l/min of a 3:1 mixture of methanol:0.5% acetic acid. Total ion signal from samples equivalent to  $10^8$  cells was obtained by scanning the range of masses corresponding to  $m/z$  values between 300 and 1500 every 2 s and then summing each of the spectra. Insets are plots of ion current for the peptide ( $M+H$ )<sup>+</sup> ions at  $m/z$  1121 (nominal mass).

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\*Contributions of these authors were equivalent and their order should be considered arbitrary.

whereas 90% were present at 30 to 150 fmol. By this measure, individual peptides in the mixture represent between 0.08 and 1.2% of the total peptide bound. Depending on the cell, this would correspond to as few as 100 complexes per cell or as many as 3000. These numbers are in accord with estimates of the number of peptide-MHC complexes required to stimulate T cells (12, 15, 16). However, 67 of the 200 peptides detected carried two-thirds of the ion cur-

rent observed for this group. Thus, HLA-A2.1 seems to be preferentially occupied by a relatively small set of peptides. On the other hand, the number of peptides detected accounts for only 50% of the total ion current. If the remaining undetected peptides are all present at amounts near that of the background, the total number of different peptides presented by HLA-A2.1 could easily exceed 1000.

Table 1 shows a partial list of data ob-

tained on peptides chosen at random from the more than 200 species observed. Fourteen of the peptides were in extracts of immunoprecipitates from both C1R-A2.1 and another HLA-A2.1-expressing cell line, JY, but not in extracts from C1R cells. Five others afforded relatively weak signals in extracts from the JY cells but were undetected in extracts from either C1R-A2.1 or C1R cells. To obtain sequence information,  $(M+H)^+$  and  $(M+2H)^{2+}$  ions were subjected to collision-activated dissociation (CAD) on the triple quadrupole mass spectrometer (17). Representative results for the  $(M+H)^+$  ion at  $m/z$  1121 (peptide 19) are shown in Fig. 2. Complete sequences for 8 peptides in Table 1 have been obtained; partial sequences have been obtained on 11 others. Each of the completely sequenced peptides is a nonamer and contains either Leu or Ile at position 2. Since Ile and Leu are of identical mass, they cannot be differentiated on the triple quadrupole instrument. Leu was considered a "dominant residue" at position 2 in unfractionated peptide mixtures isolated from HLA-A2.1 (8). For peptide 19, a definitive assignment of Leu at position 2 was established by adding synthetic peptides containing Leu or Ile at this position to the cell extract, and demonstrating that only the Leu-containing peptide coeluted from a microcapillary HPLC column with the naturally processed species. For the 19 peptides for which COOH-terminal information is available, all end in residues with aliphatic hydrocarbon side chains: nine terminate in Val, eight in Leu/Ile, and two in Ala [Table 1 and (18)]. Only Val was categorized as "dominant" based on the pool sequencing technique (8). No other residues were present at any position in more than 50% of the peptides. None of the sequences in Table 1 contains Met at position 2, Lys at position 4, Val at position 6, or Lys at position 8, assignments that were considered "strong" in analysis of pooled peptides (8).

The length of the peptides bound to HLA-A2.1 is identical to that of peptides bound to HLA-B27 (9) and inferred for other class I molecules (12). This could reflect the mechanism by which these peptides were generated, or their ability to bind with particularly high affinity (3, 19). However, the motif for peptides bound to HLA-A2.1 is different from that of peptides bound to HLA-B27. Position 2 was also the most conserved in the peptides isolated from HLA-B27, but was an Arg (9). This residue is suggested to interact with negatively charged Glu<sup>45</sup> in the B pocket of the HLA-B27 antigen binding site (3). In HLA-A2.1, this pocket is hydrophobic and is capable of accommodating a Leu or an Ile side chain

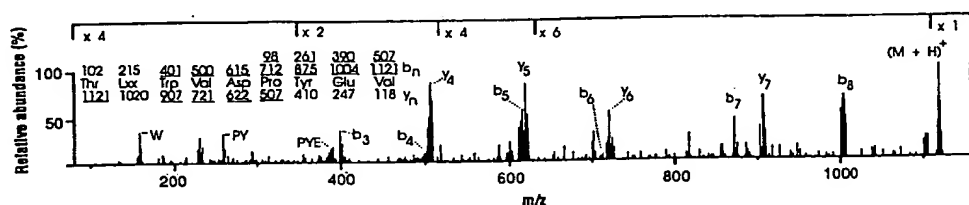
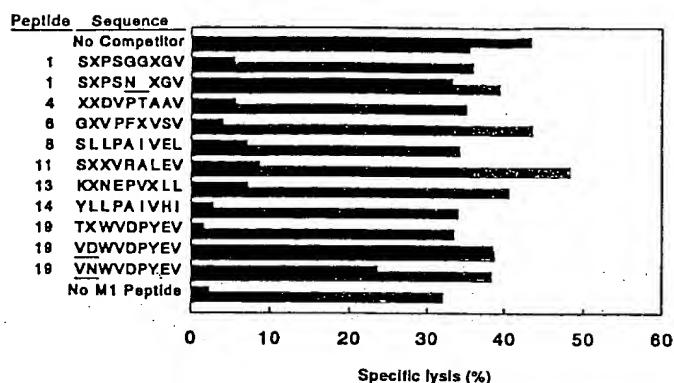


Fig. 3. Synthetic peptides corresponding to sequenced peptides bind to HLA-A2.1. Synthetic peptides are referenced by the numbers in Table 1. JY cells were incubated with the M1<sub>57-68</sub> peptide of influenza A (8 µg/ml), in the presence or absence of one of the synthetic peptides (200 µg/ml) as a competitor, in a total volume of 250 µl of RPMI 1640 containing 5% newborn calf serum and 50 µCi of <sup>51</sup>Cr for 3 hours. Washed target cells (2 × 10<sup>3</sup>) were incubated in 200 µl with either an M1 peptide-specific, HLA-A2.1-restricted CTL clone 5.2-11 (31) (filled bars) or an HLA-A2.1-specific human alloreactive CTL line (stippled bars) at an effector to target ratio of 1.25:1. Data are the means of duplicate samples with an SD of less than 5% and are representative of at least three independent experiments.



(2). In addition, positively charged residues predominate at position 9 in peptides associated with HLA-B27, and this residue was suggested to interact with Asp<sup>116</sup> in the F pocket. In HLA-A2.1, position 116 is a Tyr, rendering the F pocket smaller and more hydrophobic. Based on these considerations and the similarity of the electron density corresponding to peptide in these two molecules (2, 3), we suggest that interactions of Leu or Ile at position 2 with the B pocket, an aliphatic side chain at position 9 with the F pocket, and perhaps a nine-residue length are the primary determinants of peptide binding to HLA-A2.1.

The eight completely sequenced peptides in Table 1 were synthesized in order to demonstrate that the peptides identified actually bound to HLA-A2.1. When no complete match to a sequence in the protein or gene databases was found (below), a mixture of Leu and Ile was incorporated at positions specified by X. Cells of the HLA-A2.1-expressing line JY were incubated with the M1<sub>57-68</sub> peptide of influenza A virus in the absence or presence of a 16- to 20-fold molar excess of one of the competitor peptides and assayed for their ability to be lysed by an M1 peptide-specific, HLA-A2.1-restricted CTL clone. All eight peptides inhibited recognition under these circumstances but had no effect on recognition by a human alloreactive CTL line that is specific for HLA-A2.1 (Fig. 3). Thus, these synthetic peptides bind to HLA-A2.1 in vitro, which confirms their association with HLA-A2.1 in intact cells. Additional peptides were synthesized in which two Gly residues at positions 5 and 6 in peptide 1 were replaced by a single Asn to produce an octamer, or the Thr and Leu in positions 1 and 2 of peptide 19 were substituted with Val and Asn or Val and Asp. None of these peptides blocked recognition, indicating that a nine-residue length, the presence of

Leu or Ile at position 2, and perhaps a polar residue at position 1 are important features for peptide binding.

To identify possible precursor proteins for these peptides, gene and protein sequence databases were searched in a manner that allowed X residues to be assigned as either Leu or Ile. Only four peptides gave 100% matches with proteins in the library (Table 1). All of the sequences contain Leu at position 2, although Ile is often found at other positions. A match for peptide 8 occurs in the 65-kD  $\alpha$  and  $\beta$  regulatory subunits of human protein phosphatase 2A and in a highly homologous human transformation-associated protein, p61. Both are cytoplasmic proteins and p61 represents only 0.01 to 0.02% of the total cellular protein (20). A match for peptide 14 appears in the human nuclear protein p68, which exhibits RNA-dependent adenosine triphosphatase activity and RNA unwinding activity (21). A different sequence from this protein is found in a peptide bound to HLA-B27 (9). A match for peptide 19 is found in TIS21, a protein of unknown function that is a member of the primary response group of genes induced by growth factors (22). Finally, a match for peptide 4 is found in the human protein IP-30, the expression of which is induced by gamma interferon (23). This precursor sequence is unique among all naturally processed peptides described to date in that it is located in the signal peptide domain of the protein. This peptide is also bound to HLA-A2.1 molecules expressed on CEMx721.174(T2) (24), a mutant cell line defective in the normal antigen processing pathway (6, 7). This result suggests a second pathway through which peptides can enter the endoplasmic reticulum and associate with MHC class I molecules.

Peptides derived from the processing of normal cellular proteins located in the cytoplasm have been implicated in the correct

folding and intracellular transport of class I molecules (6, 7) and in the epitopes recognized by alloreactive T cells (25-28). The use of tandem mass spectrometry for the direct analysis of these peptides has enhanced our knowledge of peptide-MHC interactions and the cellular processes that regulate formation of these complexes and should allow the direct identification of peptides that are alloreactive T cell epitopes. Peptides associated with class II MHC molecules can also be characterized by the above approach (29). This same methodology should also facilitate identification of peptide antigens associated with viral infection, cellular transformation, and autoimmunity.

#### REFERENCES AND NOTES

1. A. Townsend and H. Bodmer, *Annu. Rev. Immunol.* 7, 601 (1989).
2. M. A. Saper, P. J. Bjorkman, D. C. Wiley, *J. Mol. Biol.* 219, 277 (1991).
3. D. R. Madden, J. C. Gorga, J. L. Strominger, D. C. Wiley, *Nature* 353, 321 (1991).
4. L. A. Morrison et al., *J. Exp. Med.* 163, 903 (1986).
5. M. W. Moore et al., *Cell* 54, 777 (1988).
6. V. Cerundolo et al., *Nature* 345, 449 (1990).
7. T. Spies et al., *ibid.* 348, 744 (1990).
8. K. Falk, O. Rotzschke, S. Stevanovic, G. Jung, H.-G. Rammensee, *ibid.* 351, 290 (1991).
9. T. S. Jardetsky, W. S. Lane, R. A. Robinson, D. R. Madden, D. C. Wiley, *ibid.* 353, 326 (1991).
10. G. M. van Bleek and S. G. Nathanson, *ibid.* 348, 213 (1990).
11. O. Rotzschke et al., *ibid.*, p. 252.
12. K. Falk et al., *J. Exp. Med.* 174, 425 (1991).
13. W. J. Storkus et al., *J. Immunol.* 138, 1657 (1987).
14. D. F. Hunt et al., in *Techniques in Protein Chemistry II*, J. J. Villafranca, Ed. (Academic Press, New York, 1991), pp. 441-454.
15. S. Demotz, H. M. Grey, A. Sette, *Science* 249, 1028 (1990).
16. E. R. Christnick, M. A. Luscher, B. H. Barber, D. B. Williams, *Nature* 352, 67 (1991).
17. D. F. Hunt et al., *Proc. Natl. Acad. Sci. U.S.A.* 83, 6233 (1986).
18. D. F. Hunt, H. Michel, J. Shabanowitz, A. Cox, unpublished data.
19. V. Cerundolo et al., *Eur. J. Immunol.* 21, 2069 (1991).
20. G. Walter, F. Ferre, O. Espiritu, A. Carbone-Wiley, *Proc. Natl. Acad. Sci. U.S.A.* 86, 8669 (1989).
21. H. Hirling, M. Scheffner, T. Restle, H. Stahl, *Nature* 339, 562 (1989).
22. B. S. Fletcher et al., *J. Biol. Chem.* 266, 14511 (1991).
23. A. D. Luster, R. L. Weinshank, R. Feinman, J. V. Ravetch, *ibid.* 263, 12036 (1988).
24. R. A. Henderson et al., *Science* 255, 1264 (1992).
25. W. R. Heath, L. A. Sherman, *Eur. J. Immunol.* 21, 153 (1991).
26. W. R. Heath et al., *Proc. Natl. Acad. Sci. U.S.A.* 88, 5101 (1991).
27. S. Man, R. D. Salter, V. H. Engelhard, *Int. Immunol.*, in press.
28. D. B. Crumpacker, J. Alexander, P. Cresswell, V. H. Engelhard, in preparation.
29. D. F. Hunt et al., in preparation.
30. P. Parham and F. M. Brodsky, *Hum. Immunol.* 3, 277 (1981).
31. V. H. Engelhard, E. Lacy, J. P. Ridge, *J. Immunol.* 146, 1226 (1991).
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5 November 1991; accepted 22 January 1992

PATENT  
0020-4491P

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Kyogo ITOH *et al.*

Serial Number: 09/202,047

Group: 1642

Filed: December 7, 1998

Examiner: Larry R. Helms

For: Tumor Antigen Proteins, Genes Therefor, and Tumor Antigen Peptides

**DECLARATION UNDER 37 C.F.R. § 1.132**

Dear Sir:

The undersigned, Kyogo Itoh, M.D., Ph.D., hereby declares and states as follows:

1. I am the same Itoh who executed the Declaration filed in the above-identified application on June 8, 2001 (referred to herein as "my earlier Declaration".)
2. I have read and understood the Office Action mailed on August 22, 2001, in which the Examiner stated that my earlier Declaration is not persuasive and pointed out that the said Declaration did not state that the protein of Nakao *et al.* in fraction number 23 is not that of amino acid sequence of SEQ ID NO; 2 in the instant application and that it is immaterial how the protein was obtained such as "expression cloning technique" because the claims are directed to the product not the method of isolation.

3. In response to the Examiner's objection, I would like to clarify that the protein of Nakao *et al.* in fraction number 23 (or any other fraction) is not that of amino acid sequence of SEQ ID NO: 2 in the instant application.

3-1. With regards to tumor antigen protein and tumor antigen peptide

As described in "PRIOR ART" of the present specification, tumor antigen peptides are generated from tumor antigen proteins, which have been synthesized within a cell, through the intracellular decomposition by proteasome. The resultant tumor antigen peptides bind to MHC class I antigens to form a complex intracellularly and presented on the cell surface. CTLs only can recognize thus presented complex of tumor antigen peptide-MHC class I antigen.

Accordingly, the "tumor antigen protein" is present only within cells while the "tumor antigen peptides" consisting of 8-12 amino acids, which are decomposition product of the protein, are generated within the cell but keep staying on the cell surface in the form of a complex with MHC class I antigen.

3-2. Nakao *et al.*

An enormous number of antigen peptides (including tumor antigen peptides) generated from various antigen proteins are coexisting on the surface of carcinoma cell line KE-4.

Nakao *et al.* eluted the peptides on the cell surface by pH3.3 acid elution technique and subjected the extract to pretreatment with C18 Sep Pak. Nakao *et al.* then conducted prefractionation to obtain peptides having molecular weight of up to 3000 (about 30 amino acids or less), thereby removing the contaminants. The resultant prefractionation product consisting of peptides having molecular weight of 3000 or less was subjected to reverse HPLC as shown in Fig. 3. It is apparent that the sample used in the HPLC contained peptides having molecular

weight of 3000 or less and not the tumor antigen protein of the present invention of 800 amino acids.

This can be confirmed by referring to the description on page 4248, right column, "Peptides and Tumor Cells" of Nakao *et al.*, Cancer Research 55, 4248-4252. The description of Fig. 3 which states "HPLC fraction of the KE peptides" also support the above.

Further, the assay system used in Nakao *et al.* for determining the activity of HPLC fractions is effective for the identification of tumor antigen peptides, which is not applicable to tumor antigen proteins. Accordingly, a substance having identified to be active must be "a tumor antigen peptide(s)".

As mentioned above, the Fraction 23 was obtained through HPLC of a sample consisting of peptides having molecular weight of 3000 (about 30 amino acids) or less extracted from a cell, and hence could not contain a tumor antigen protein of the present invention consisting of 800 amino acids. Accordingly, Nakao *et al.* never disclosed the tumor antigen protein of the present invention.

As explained in my earlier Declaration, fraction No. 23 or other fractions of Fig. 3 of Nakao *et al.* consists of a number of peptides whose structure or origin is unknown. It is generally accepted in the art to which the present invention pertains that one fraction obtained by a method such as that described in Nakao *et al.* must contain as many as about one hundred kinds of peptides. It was practically impossible to isolate and determine the structure or origin of a given peptide among such a huge number of peptides originated from unknown antigens by following the teaching of Nakao *et al.* The present inventors could not succeed in the cloning of tumor antigen protein of the present invention until the "expression cloning technique" was at last employed.

In addition, the purpose of the research of Nakao *et al.* was to confirm the


Kyogo Itoh, M.D., Ph.D.

existence of HLA-A2601 restricted CTLs in cancer patients, which means that they did not intend to isolate any novel tumor antigen protein from a fraction shown in Fig. 3, as can be seen from the description in "Abstract" on page 4248.

4. In summary, it is my conclusion that Nakao *et al.* does not disclose a tumor antigen protein of the present invention having amino acid sequence of SEQ ID NO: 2.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Date: December 7, 2001

  
Kyogo Itoh, M.D., Ph.D.